To the Graduate Council:

I am submitting herewith a thesis written by Susan Gurwitch entitled "Analysis of Gene Expression During Prophase I of Meiosis". I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Masters of Science, with a major in Life Sciences.

Mary Ann Handel

Mary Ann Handel, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of the Graduate School

ANALYSIS OF GENE EXPRESSION DURING PROPHASE I OF MEIOSIS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Susan Gurwitch

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To the past and previous members of the Handel lab, Cynthia Park, Dr. Laura Richardson, Dr. Tim Wiltshire, Dr. Kim Caldwell, John Cobb, and many undergraduate students, thank you for all you have done for me.

Last, I would like to thank my friends and family: my parents, Bert and Janet Gurwitch and of course, my best friend, Geoff Rutledge. Thank you all for being there for me.

ABSTRACT

Although meiosis is an important process, little is known about gene expression during this time. Therefore, cDNA libraries from specific populations of male mouse meiotic germ cells were subjected to a differential screening process that made use of genetic mutants to identify sequences that are presumed to be expressed in meiotic prophase (Caldwell et al., 1996. Molec Reprod Devel: 43:403-413). Analysis of sequences by BLAST revealed that of the 18, 9 were identical or closely homologous to known protein coding sequences (including calmodulin, carnitine palmitoyl transferase I-like protein, ILGF binding protein, and the mouse *Rbm* gene that is highly homologous to the human AZF spermatogenic factor). The remainder were novel sequences not in the sequence data bases. Each cDNA clone was used to probe both tissue and germ-cell specific northern blots to determine specific patterns of expression. Two clones identified by this screen, *Ott* and *Rbm*, are sex-linked genes, and were not expected to be expressed during this time in meiosis. Because they were identified by this screen, they became interesting targets for further analysis. Therefore more detailed expression analysis was done using these two clones. Here we report the results of the expression analysis of the 18 clones, as well as more detailed analysis of *Ott* and *Rbm*.

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CHAPTER I: Introduction

Spermatogenesis, the process by which diploid cells become haploid gametes in males, includes three phases: mitotic expansion of the stem cell pool, meiosis, and post-meiotic spermiogenic differentiation. The focus of this study is the meiotic phase. Prophase I of the meiotic phase is the longest stage of meiosis. Prophase I consists of five substages: leptotene, zygotene, pachytene, diplotene, and diakinesis. During the leptotene substage chromosome condensation is initiated. The zygotene substage is marked by initiation of formation of the synaptonemal complex, which is thought to hold homologous chromosomes together and aid in recombination. The pachytene substage, the lengthiest part of prophase I, includes pairing of homologous chromosomes, complete formation of synaptonemal complexes, and recombination. After the pachytene substage, cells enter into metaphase I (MI), when the synaptonemal complex disassembles, chromosomes condense, the nuclear envelope breaks down, the spindle forms, and paired chromosomes joined by chiasmata (the manifestation of prior recombination) align on the spindle. The first division then occurs with separation of homologous chromosomes. The second division follows rapidly with separation of sister chromatids resulting in haploid cells. These haploid cells then proceed through spermiogenesis and eventually become fully mature sperm.

Meiosis is a very important process for several reasons. For example, if meiosis were not to occur properly, the result could be infertility. In addition, errors in this process can lead to chromosomally unbalanced gametes, causing aneuploidy, and associated genetic disorders in offspring. Finally, meiosis is the process which controls the inheritance of traits producing genetic diversity within a population.

Spermatogenesis occurs throughout the adult life of male mammals; therefore the process of mammalian meiotic prophase is more easily studied in males than in females. In females, meiotic prophase occurs during fetal development, and is arrested until puberty. The cytogenetics of meiotic prophase have been studied more extensively in male mammals, where these events are more accessible.

The mouse model is advantageous to use for analysis of meiosis because the developmental biology of testicular development has been well studied, and methods have been developed to isolate testes at different ages so that only certain germ cells are present. Cell separation techniques have also been developed that allow separation of only specific germ cell types (Bellvé et al., 1977). Also, a number of genetic aberrations that affect meiotic stages of spermatogenesis have been discovered (reviewed by Handel, 1990). These mutations not only allow genetic screening, but may eventually aid in determining exact sequences of events within spermatogenesis.

The long term goal of this work is to identify genes important for meiosis.

The immediate purposes of this study were 1) to analyze sequence and expression of cloned sequences that had been identified in a differential screen, and 2) to study a subset of those in more detail because, unexpectedly, they were sex-chromosome encoded sequences. Informatics databases were used for sequence analysis. Analyzing gene expression by Northern analysis was the primary method of investigating patterns of expression. Use of somatic tissue to examine tissue specificity of these clones permits detection of any testis-specific genes, and our ability to isolate germ cells from the testis enabled us to evaluate gene expression throughout prophase I of meiosis.

Determining genes that are expressed during spermatogenesis may lead to a better understanding of the process as a whole, as well as to insights into male sterility and causes of aneuploidy.

CHAPTER II: Gene Sequences Expressed in Meiosis

BACKGROUND AND SIGNIFICANCE

Although the formation of sperm is an important process, little is known about gene expression during the meiotic stage of spermatogenesis. Therefore determining genes which may be expressed specifically in each stage of prophase I may aid in clarifying the processes occurring in each substage of meiosis. We initiated this by ascertaining genes expressed in the window of time between the leptotene-zygotene (L/Z) and pachytene (P) substages of meiosis I prophase (Caldwell *et al.*, 1996).

Genes expressed specifically during meiosis can be assessed in two ways. The first is to identify sequences in mouse with homology to sequences known to be expressed during meiosis in other sexually reproducing processes. Because meiosis is such a conserved process, it is believed that genes expressed in one species during this time will also be expressed in other sexually reproducing species. The second approach is to use the technique of differential screening to identify sequences expressed in meiosis during spermatogenesis (Caldwell *et al.*, 1996). Determination of genes expressed during this time, which may be expressed only during this process, may lead to the understanding of spermatogenesis as a whole.

Previously, cDNA libraries were created from leptotene/zygotene

spermatocytes (L/Z), pachytene spermatocytes (P), round spermatids (RS), and whole testis (WT) cells. The L/Z and P libraries were then subjected to a novel subtractive hybridization screening process (Caldwell *et al.*, 1996) that made use of two translocation mutants in the mouse testes: T38, which halts spermatogenesis at the L/Z stage, and T16, which halts spermatogenesis at late pachytene. cDNA from testes of T38 mice was used to first screen the libraries, and nonhybridizing colonies were selected. This screen was repeated to ensure that no false negatives were retained. These colonies theoretically represent cDNA expressed after the halt at the L/Z stage. They were next screened with cDNA from the testes of T16 mice and in this screen positively hybridizing colonies were selected (Figure 2.1). These clones represent genes that are presumably expressed in the window of time between L/Z and late P. They may also represent genes that are necessary for progression through the pachytene stage.

By this method, Caldwell *et al.* (1996) identified 18 clones that did not hybridize to cDNA from T38 testes but did hybridize to cDNA from T16 testes. Prior to the present study, these clones were subjected to single pass automated sequencing. In this study their partial sequences were subjected to BLAST analysis. Of the 18 clones, 10 were found to be homologous to known proteins and the remaining 8 were apparently novel. Each clone was then used as a probe on cell-specific and tissue-specific Northern blots to ascertain their patterns of expression during spermatogenesis.

Screening Strategy Using T38 And T16 Translocations

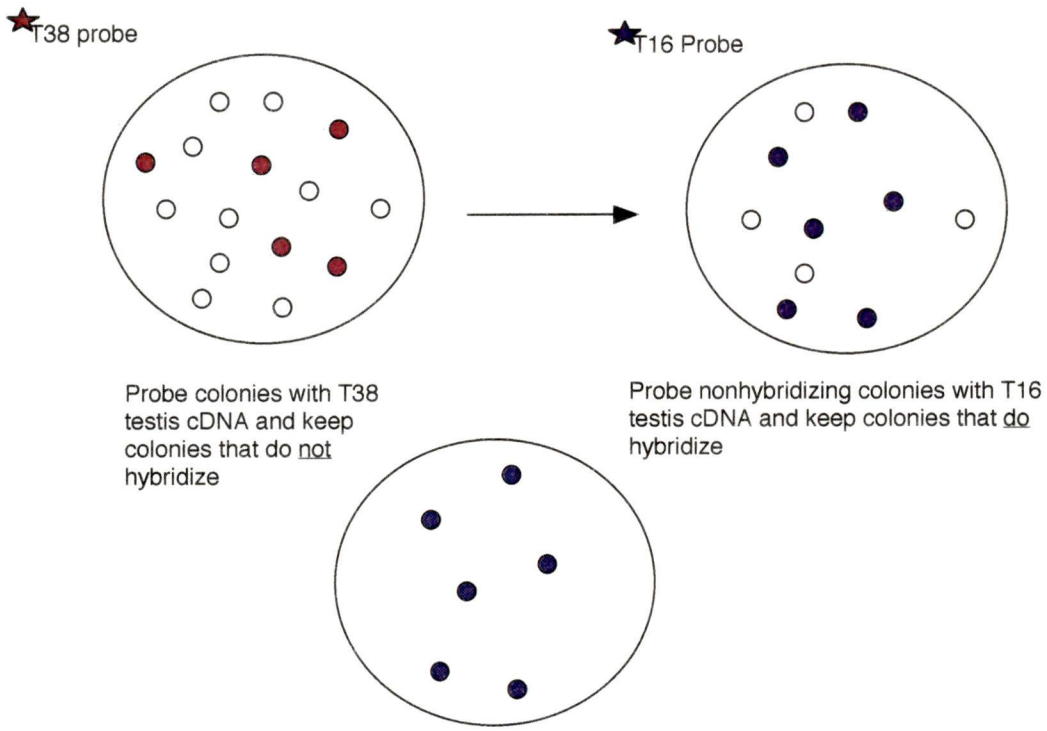


Fig. 2.1 - Screening strategy used to identify cDNA clones proposed to be expressed in the window of time between the L/Z stages and late P stage of meiotic prophase.

MATERIALS AND METHODS

Isolation of Spermatogenic Cells

Germ cells were prepared from 8-day- old mouse testes (for type A and type B spermatogonia), from 17-day-old mouse testes (for L/Z cells), and from adult mouse testes (to yield adult P spermatocytes, RS, and residual bodies (RB)). Testis tubules were suspended in KRB [120.1 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 1.3 mM $CaCl_2$, 11 mM glucose, 1X essential amino acids (Sigma, St. Louis, MO), 1X non-essential amino acids (Sigma, St. Louis, MO)] and were digested with collagenase, trypsin and DNase as previously described (Romrell *et al.,* 1976; Bellvé *et al.,* 1977). After tubule digestion, cells were washed with 0.5% bovine serum albumin to remove trypsin and collagenase. Cells were then separated by sedimentation on a bovine serum albumin gradient at unit density using the STA-PUT chamber (Johns Scientific, Ontario). After 2.5 hours, cells were collected in 10 ml fractions at a rate of 10 ml/43 seconds. Cell fractions were assessed for morphology and purity by differential interference contrast (DIC) optics and enriched fractions pooled. Each cell type was enriched to a purity of >80%. Type A and type B spermatogonia, as well as leptotene/zygotene spermatocytes were obtained using 65 mice of the appropriate age. Pachytene spermatocytes, round spermatids, and residual bodies were isolated using 10 adult mice.

Isolation of Total RNA from Germ Cells

Total RNA was isolated from germ cells by the method of Cathala *et al.* (1983). Diethylpyrocarbonate-treated glassware and solutions were used in all steps. Purified germ cells were frozen at -80° overnight, and were then thawed in guanidinium isothiocyanate (5 M guanidinium isothiocyanate, 10 M EDTA, 50 mM Tris-HCl pH 7.5) and sheared with a 20 1/2 gauge syringe 20 times. The RNA was precipitated overnight with 7 volumes 4 M lithium chloride at 4°. Samples were then centrifuged at 4° for 1.5 hours at 10,000 x g and the pellets were resuspended in RNA solubilization buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% SDS). RNA was extracted with phenol followed by chloroform then precipitated with sodium acetate (0.2 M NaAc) and 100% ethanol overnight. Samples were then centrifuged for 10 minutes at high speed in the microcentrifuge and the pellets were resuspended in 200 µl depc-water and were quantitated by spectrophotometry. In addition to preparing RNA from specific germ cell types, RNA was isolated by the same methods from whole testes of adult mice (WT) and from testes of genetically germ-cell-deficient mice (XX, *Sxr*). Mouse somatic tissue RNA was obtained from Ambion (Austin, TX).

Northern Analysis

15 µg of total RNA from each sample (XX*Sxr*, WT, A, B, LZ, P, RS, and RB) was denatured in 50% formamide (Ambion, Austin, TX), 1X MOPS buffer, 6.5% formaldehyde pH 6.0, and Type III loading buffer [0.25% bromophenol

blue, 0.25% xylene cyanol, and 30 % glycerol (Sambrook *et al.*, 1982)] for 15 minutes at 65°. The samples were then loaded onto a denaturing 1% agarose gel (1X MOPS buffer, 2.2 M formaldehyde). Samples were electrophoreses at 50V for 4.5 hours or until the dye had moved about 3/4 of the gel length. The gel was then washed twice in deionized water for 30 minutes, stained with ethidium bromide at 5μg/ml for 20 minutes, and destained for 20 minutes. The gel was photographed to visualize the ribosomal 28S and 18S bands. It was then soaked in 20X SSC (3M NaCl, 0.3M NaCitrate) and transferred overnight to nylon membrane (Sratagene, LaJolla, CA). RNA was crosslinked to the nylon blot at 1280 μjoules/cm^2 in a Stratalinker (Stratagene, LaJolla, CA).

Hybridization of Northern Blots

Prehybridization was in 5X SSPE, (3M NaCl, 0.02M EDTA, 0.1M NaH_2PO_4), 5X Denhardt's Solution (6.25nM Ficoll, 62.5nM polyvinylpyrrolidone, 1% BSA), 0.5% SDS, 0.25 mg/ml fish sperm DNA (Boehringer Mannheim, Indianapolis, IN) at 65°C for 3 hours in a hybridization incubator (Lab-Line Industries). After removal of the prehybridization solution, fresh prehybridization solution was added with denatured probe at a concentration of 1 million cpm/ml. Probes were made from clones in Bluescript plasmid (Stratagene, LaJolla, CA); standard methods were used for isolating the inserts and radiolabelling by the random-priming method with α-32PdCTP (ICN, Costa Mesa, CA). Hybridization was at 65°C overnight in the

incubator. Following hybridization the blots were washed twice with 1X SSC and 0.1% SDS at 65°C and the twice with 0.1X SSC at 65°C. Blots were wrapped in plastic and exposed to film (Reflections, ICN, Costa Mesa, CA) at -80°C for appropriate period of time. Stripping of blots was done using boiling SDS (0.25%) as recommended by the manufacturer (Stratagene).

Isolation of Plasmid Clones and Sequence Analysis

Clones were purified using Nucleobond AX-100 purification columns (The Nest Group, Macherey-Nagel). Aliquots of each clone were subjected to single-pass automated sequencing, conducted under the direction of Dr. J. Schimenti, The Jackson Laboratory. This yielded at least partial sequence for each clone. Comparison to sequence data bases was performed using the BLAST algorithm (National Center for Biotechnology Information). BLAST analysis was performed on a regular basis throughout the study.

RESULTS

Sequence Analysis

BLAST analysis of sequences revealed many of the clones isolated were homologous or identical to known proteins (Table 2.1). These are calmodulin, *Rbm* (RNA- binding motif), *Ott* (Ovary-testes transcribed), thymosin beta, alpha tubulin, gene *Ews* protein, carnitine palmitoyl transferase, alanyl tRNA

Table 2.1: Summary of Expression Analysis

Table 2.1 - Summary of expression analysis. All cDNA clones represent sequences presumed to be present in spermatogenic cells during the time from early meiotic prophase to mid-to-late prophase (Caldwell *et al.*, 1996). The cell-specific and tissue-specific Northern blot analysis data are given. WT-whole testis, XX-XX,*Sxr* germ-cell deficient testes, AB-type A and B spermatogonia, LZ-leptotene/zygotene spermatocytes, P-pachytene spermatocytes, RS-round spermatids, RB-residual bodies, B- brain, E-embryo, H-heart, K-kidney, L-liver, Lu-lung, O-ovary, S-spleen, T-testes, and Th-thymus. Letters and colors represent intensities of bands on blots. H-heavy, M-medium, L-low, N.D.- no data available.

1. Homology determined by BLAST analysis
2. Highly homologous to known sequences

Clone Number	Sequence Homology of cDNA Clones[1]	Cell-Specific Northern Blot Results							Tissue-Specific Northern Blot Results									
		WT	XX	AB	LZ	P	RS	RB	B	E	H	K	L	Lu	O	S	T	Th
1	Novel	L			L	H	H	L						N.D.			L	
2	Carnitine palmitoyl transferase I-like protein	M				M	M		L	L	L	L	L	L	L	L	L	L
3	Alanyl tRNA synthetase	M	L	M	L	H	M		M		L	L	L	L	L	M	L	
4	Calmodulin[2]	H	M	M	M	H	H	L	H	M		L			M	M	M	M
6	Novel	M				M	M	M	L								L	
7	No sequence	M				H	H	M	M	L		L			L	L	M	L
8	Novel	M			L	H	M										L	
9	Gene Ews protein[2]	H	L	M	L	H	H		L	L	L	L		N.D.	L	L	L	M
10	ILGF binding protein[2]	L	L	M	H	L	L		L	L	L	L	L	L	L	L	L	L
11	Mouse *Rbm*	H		M	L	H	M		L	L	L	L	L	L	L	L	L	L
12	Ovary-testes transcribed (*Ott*)					M											M	
13	Novel																L	
14	Thymosin Beta[2]	H	L	M	L	H	H	M	M	H	L	L		N.D.	M	M	L	H
15	Gene Ews Protein[2]	H	L	M	L	M	M		L	L	L	L			L	L	L	M
16	Novel	M	L	M	L	M	M		L	L		L		N.D.	L	L	L	H
17	Novel	L				M	M										L	
18	Alpha tubulin[2]	L	L	L	L	L	L		M	M	L	L	L	N.D.	L	L	L	M
19	Similar to C. *elegans* carrier protein	L	L	L	L	M	M	L	L	L	L	L	L	L	L	L	L	L

synthetase, and ILGF (Insulin-like growth factor). Eight of the 18 clones isolated had sequence not homologous to others in the BLAST data-base analysis, and therefore appear to be novel gene sequences.

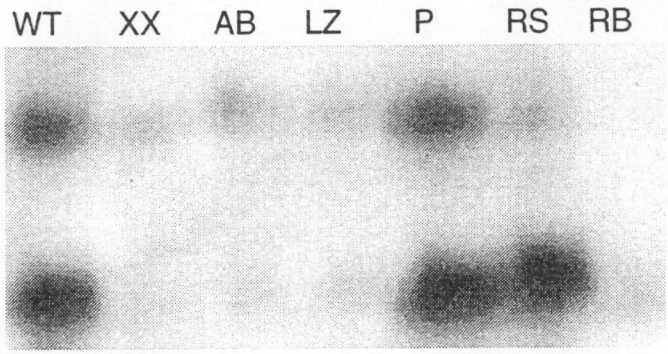
Expression Analysis

A summary of results from Northern analysis of both cell-specific and tissue-specific blots is shown in Table 2.1. All clones resulted in banding patterns on both testis-specific and other tissue Northern blots.

The expression patterns of obtained from each clone are shown. These are calmodulin (Fig. 2.2), thymosin beta (Fig. 2.3), alpha tubulin (Fig. 2.4), gene *Ews* protein (Fig. 2.5), carnitine palmitoyl transferase (Fig. 2.6), alanyl tRNA synthetase (Fig. 2.7), and ILGF (Fig. 2.8), *Rbm* (Fig. 2.9), and *Ott* (Fig. 2.10). Expression patterns of two novel clones are shown in Fig. 2.11.

We categorized the diverse expression patterns revealed by these blots into four different groups: testis-specific expression, testis and brain expression, expression not restricted to germ cells, and ubiquitous expression (Table 2.2). The most interesting group for future analysis is the testis-specific category. Four of the clones in this set are novel. The banding patterns for these samples are similar, most showing expression in L/Z, P, and RS. Finding four novel testis-specific clones is promising in that they may be involved in some aspect of meiosis, or more likely in spermiogenesis since their expression is late in prophase.

A.



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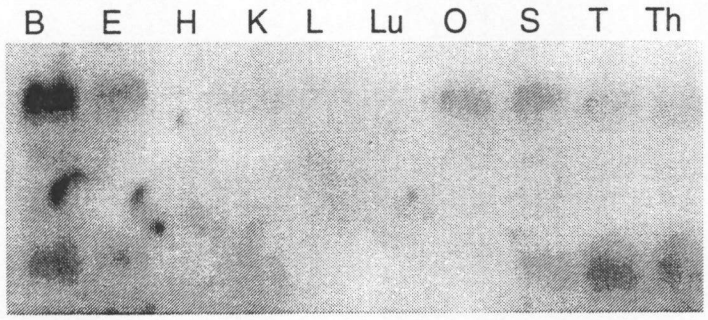
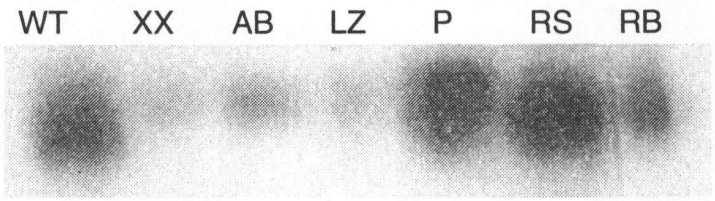


Fig. 2.2 - A. Cell-specific and B. tissue-specific Northern blots of calmodulin sequences. WT- whole testes; XX - XX,*Sxr;* AB - A and B spermatogonia; LZ - leptotene/zygotene spermatocytes; P - pachytene spermatocytes; RS - round spermatids; RB- residual bodies; B - brain; E - embryo; H -heart; K - kidney; L - liver; Lu - lung; O - ovary; S - spleen; T - testes; Th - thymus

A.



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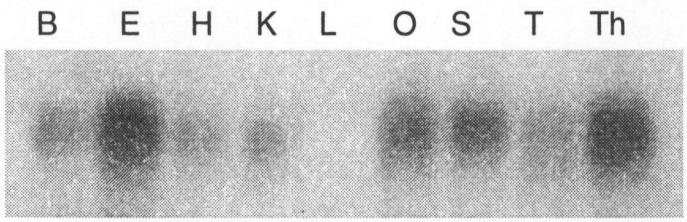
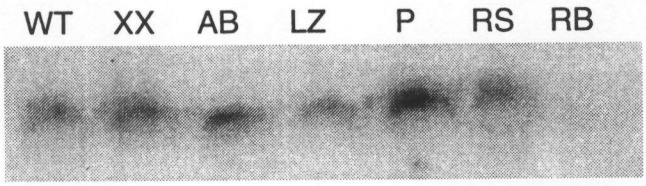


Fig. 2.3 - A. Cell-specifIc and B. tissue-specific Northern blots of thymosin beta. WT-whole testes; XX - XX,*Sxr;* AB - A and B spermatogonia; LZ - leptotene/zygotene spermatocytes; P - pachytene spermatocytes; RS - round spermatids; RB- residual bodies; B - brain; E - embryo; H -heart; K - kidney; L - liver; O - ovary; S - spleen; T - testes; Th - thymus

A.



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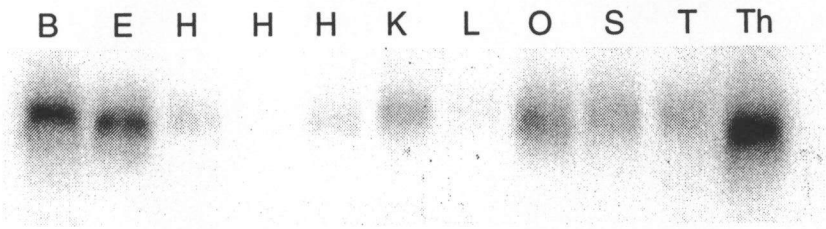


Fig. 2.4 - A. Cell-specific and B. tissue-specific Northern blots of alpha tubulin. WT-whole testes; XX - XX,*Sxr;* AB - A and B spermatogonia; LZ - leptotene/zygotene spermatocytes; P - pachytene spermatocytes; RS - round spermatids; RB- residual bodies; B - brain; E - embryo; H -heart; K - kidney; L - liver; O - ovary; S - spleen; T - testes; Th - thymus. When loading this gel, the heart RNA leaked across three lanes; thus each one is labeled.

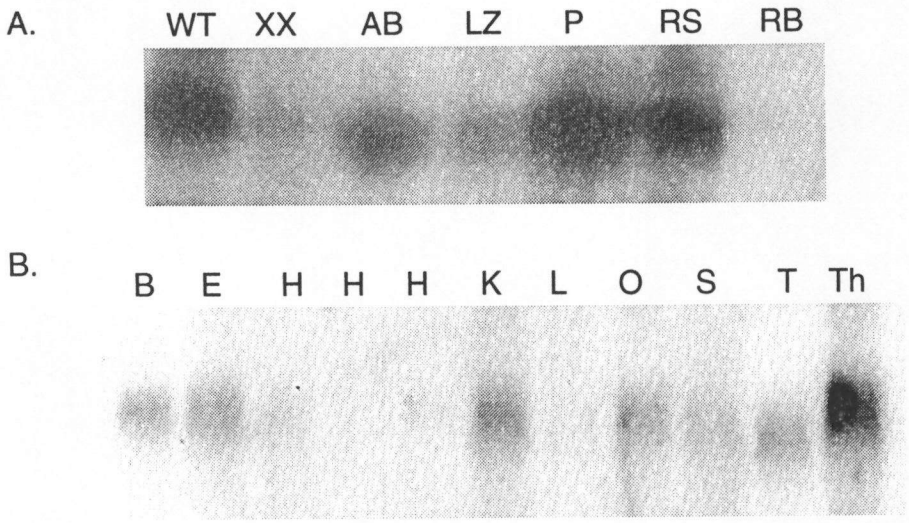
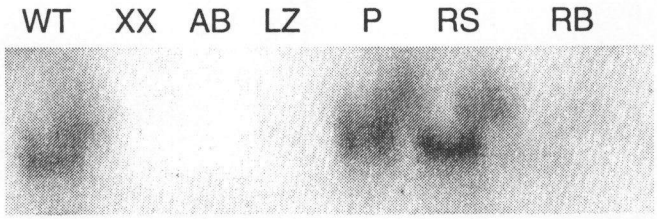


Fig. 2.5 - A. Cell-specifIc and B. tissue-specific Northern blots of gene *Ews* protein. WT-whole testes; XX - XX,*Sxr;* AB - A and B spermatogonia; LZ - leptotene/zygotene spermatocytes; P - pachytene spermatocytes; RS - round spermatids; RB- residual bodies; B - brain; E - embryo; H -heart; K - kidney; L - liver; O - ovary; S - spleen; T - testes; Th - thymus. Heart Lanes leaked when gel was loaded, therefore in 3 lanes.

A.



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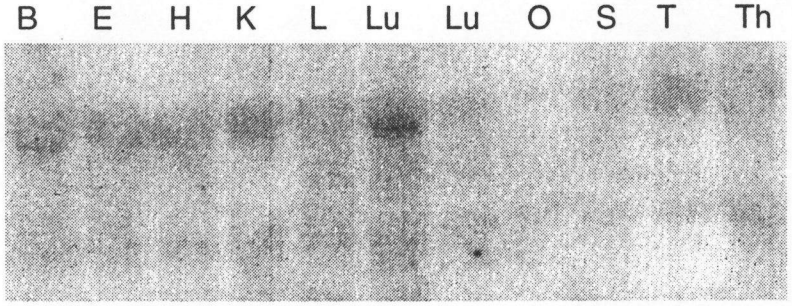


Fig. 2.6 - A. Cell-specific and B. tissue-specific Northern blots of carnitine palmitoyl transferase. WT-whole testes; XX - XX,*Sxr;* AB - A and B spermatogonia; LZ - leptotene/zygotene spermatocytes; P - pachytene spermatocytes; RS - round spermatids; RB- residual bodies; B - brain; E - embryo; H -heart; K - kidney; L - liver; Lu - lung; O - ovary; S - spleen; T - testes; Th - thymus. Lung was loaded in 2 lanes.

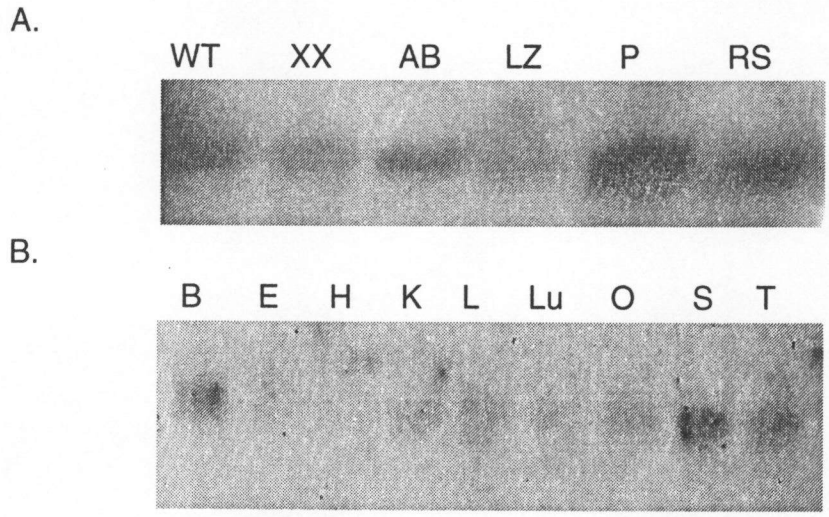
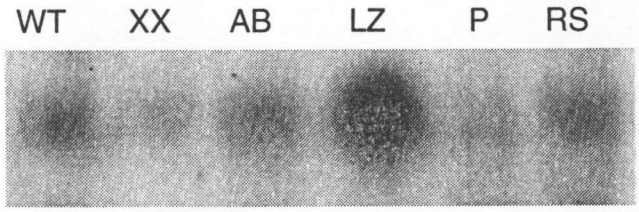


Fig. 2.7 - A. Cell-specific and B. tissue-specific Northern blots of alanyl tRNA synthetase. WT-whole testes; XX - XX,*Sxr;* AB - A and B spermatogonia; LZ - leptotene/zygotene spermatocytes; P - pachytene spermatocytes; RS - round spermatids; B - brain; E - embryo; H -heart; K - kidney; L - liver; Lu - lung O - ovary; S - spleen; T - testes.

A.



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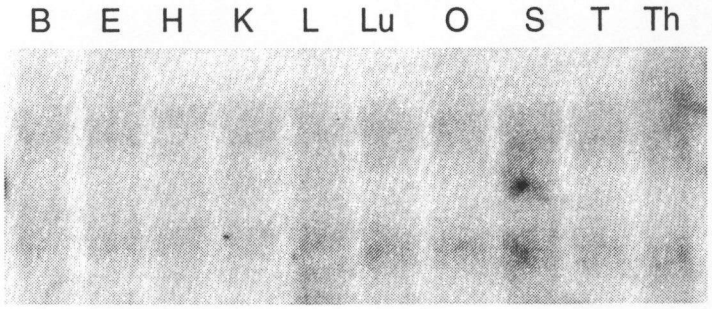
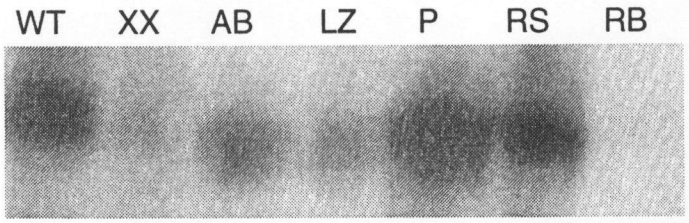


Fig. 2.8 - A. Cell-specific and B. tissue-specific Northern blots of ILGF. WT-whole testes; XX - XX,*Sxr;* AB - A and B spermatogonia; LZ - leptotene/zygotene spermatocytes; P - pachytene spermatocytes; RS - round spermatids; B - brain; E - embryo; H -heart; K - kidney; L - liver; L - lung; O - ovary; S - spleen; T - testes; Th - thymus.

A.



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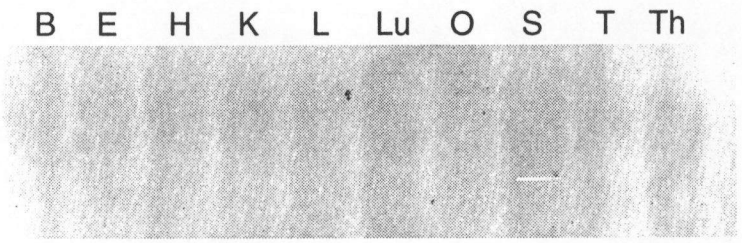


Fig. 2.9 - A. Cell-specific and B. tissue-specific Northern blots of *Rbm*. WT-whole testes; XX - XX,*Sxr;* AB - A and B spermatogonia; LZ - leptotene/zygotene spermatocytes; P - pachytene spermatocytes; RS - round spermatids; RB- residual bodies; B - brain; E - embryo; H -heart; K - kidney; L - liver; Lu - lung; O - ovary; S - spleen; T - testes; Th - thymus.

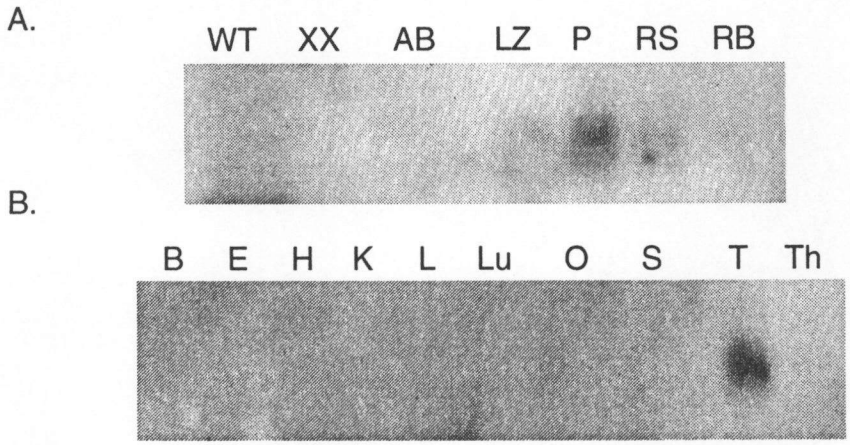
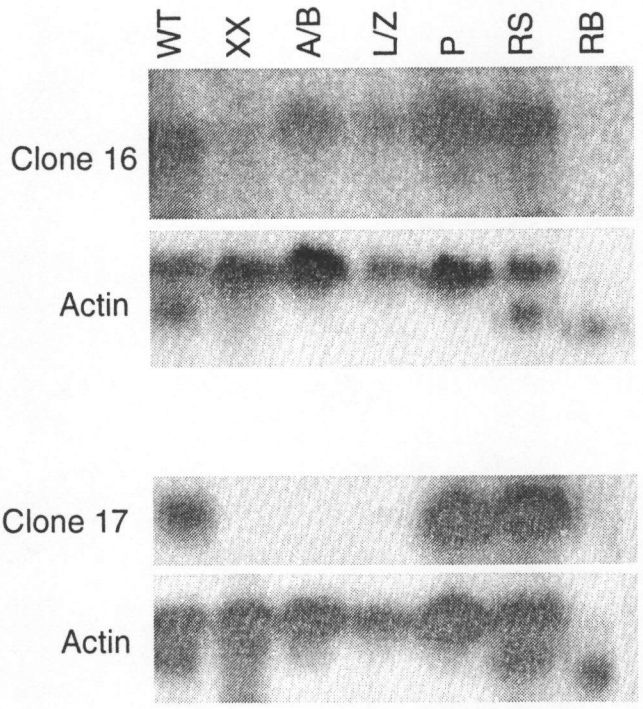


Fig. 2.10 - A. Cell-specific and B. tissue-specific Northern blots of *Ott*. WT-whole testes; XX - XX,*Sxr;* AB - A and B spermatogonia; LZ - leptotene/zygotene spermatocytes; P - pachytene spermatocytes; RS - round spermatids; RB- residual bodies; B - brain; E - embryo; H -heart; K - kidney; L - liver; Lu - lung; O - ovary; S - spleen; T - testes; Th - thymus.

Fig. 2.11 - Representative Northern analyses of novel sequences identified by the screening process. A. Cell-specific and B. tissue-specific autoradiographs. Actin control blots are shown with each. WT - whole testis; XX - XX,*Sxr;* AB - A and B spermatogonia; LZ - leptotene/zygotene spermatocytes; P - pachytene spermatocytes; RS - round spermatids; RB- residual bodies; B - brain; E - embryo; H -heart; K - kidney; L - liver; Lu - lung; O - ovary; S - spleen; T - testes;
Th - thymus.

A.



B.

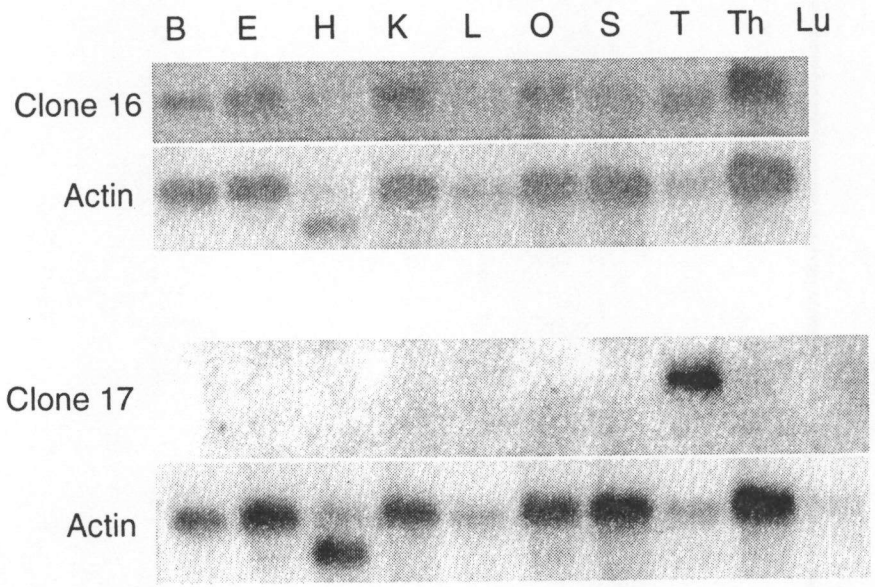


Table 2.2 - Categories of Expression Patterns of the 18 Clones

Category	Clone Number(s)	Expression
Testis-Specific Expression	1,8,12,13,17	In WT, LZ, P, and RS; #1 also expressed in RB (no germ-cell data for #13)
Testes and Brain Expression	6	In WT, P, RS, RB, and brain
Expression Not Restricted to Germ Cells	3,4,7,9,14,15,16	In many tissues other than testes; for many also expressed in XX*Sxr* germ-cell-deficient testes
Expression Ubiquitous	2,10,11,18,19	In all tissues tested

Table 2.2 - The expression patterns of the 18 clones were divided into four different categories; testis-specific, testes and brain, not restricted to germ cells, and ubiquitous.

The next category, expression restricted to the testis and brain includes only one clone, number 6. This is a novel sequence that is expressed in WT, P, RS, and RB, as well as brain.

The category "not restricted to germ cells" encompasses six of the clones isolated. All of these clones detected expression in tissues other than testis, and many of them also revealed expression in XX*Sxr* testes, which are germ cell deficient.

The last category of clones, ubiquitous expression, is comprised of five clones. These include alpha tubulin, ILGF, carnitine palmitoyl transferase, and *Rbm.*

DISCUSSION

The main goal of previous work (Caldwell et al., 1996) was to identify genes expressed during the window of time between the leptotene/zygotene and late pachytene stages of meiosis during spermatogenesis. In this specific project we first analyzed sequences to identify clones isolated in this novel screening process. We then examined expression patterns of sequences recognized by the clones on both cell-specific and tissue-specific Northern blots. We were able to identify 10 of the 18 clones as being homologous to known genes and 7 as novel (for one we had no sequence). Northern analysis revealed different patterns of expression allocated into 4 categories: testis-specific, testis and brain, not

restricted to germ cells, and ubiquitous. This work reveals the complexity of gene expression during spermatogenesis, but hopefully ultimately it may lead to better understanding of the process.

Below we consider the cloned sequences in categories defined by the results of sequence analysis.

House-keeping Genes

It was surprising to identify house-keeping genes by this screen. The method was designed to isolate genes uniquely expressed between L/Z and late P, and it was thought that the screen would eliminate genes ubiquitously expressed in germ cells of both T38 and T16 mice. We did however retrieve gene sequences, such as calmodulin, known to be involved in many different processes. Thus it is apparent that the screen did not effectively subtract sequences with non-specific expression patterns or that there is, for some reason, very high expression of some house-keeping sequences during the pachytene stage. I discuss below the sequences identified in this category.

Calmodulin is an intracellular calcium receptor that has been implicated as an essential protein in amphibian meiosis; this is less firmly established in mammals. In rat testes, transcripts from at least three genes encoding the exact same calmodulin protein have been found however, in the blots shown here only two bands are seen. The use of multigene families is one way to ensure continued presence of important proteins at critical times. This may explain why there is

more than one calmodulin gene expressed in the rat testis. Different calmodulin gene products might influence meiosis at multiple points by responding to different transacting regulators present during different times of prophase I. Our data show a lower molecular weight band apparently expressed most strongly in the testis, although it is expressed also in the brain and thymus. This product is also expressed most dominantly in the P and RS lanes on the cell-specific blot. This is very interesting because this may indicate that there is a specific calmodulin isoform that might be influencing meiosis at this time.

Calmodulin is a regulator of many protein kinases, and phosphorylation by kinases is implicated as a cell cycle control regulator which may be why we see an increase at the pachytene stage. Another target of calmodulin could be microtubule networks and actin filaments involved in chromosomal separation during meiosis. Polymerization states of both of these proteins are controlled by calmodulin (Slaughter and Means, 1989). Thus there could be many possible meiosis-specific roles for calmodulin during spermatogenesis. However, calmodulin could also be present here as a house-keeping gene, aiding in the normal metabolic and signalling processes of the cell.

The function of the thymosin β_{10} protein has not been completely elucidated, but the complete conservation of sequence across many species suggests that this protein plays an important role in vertebrate biology. Thymosins in general act as actin monomer binding proteins, inhibiting polymerization of actin filaments. It has been shown by Lin and Morrison-

Bogorad (1991) that thymosin β_{10} mRNA is found in spermatocytes, but the protein does not appear until round spermatids. Thus we have expected, and see confirmed, that some "meiosis" gene expression is really not for meiosis but is making mRNA templates to be used in spermiogenesis.

Tubulin, another sequence identified by this screen, is believed to play important roles in meiotic (as well as mitotic) divisions, in the process of changes in cell shape and structure, in formation of species-specific shapes of sperm heads, and in structure and function of the axoneme of the sperm tail. There are two types of tubulins; the beta tubulin is expressed early in meiosis, whereas the alpha tubulin is expressed later during spermatogenesis. Therefore finding alpha tubulin as one of our clones was not surprising. Since alpha tubulin expression is increased during spermiogenesis, it is likely that it plays a larger role later in spermatogenesis, possibly in the nuclear shaping or assembly of the axoneme.

Ews is a RNA binding-domain protein, implicated in the formation of tumors in Ewing sarcoma (Plougastel et al., 1994), that was identified by this screen. Its possible function in meiosis is unknown, nonetheless, it is expressed in pre- and post-meiotic germ cells.

Carnitine palmitoyl transferase was also identified as expressed by this screen. Its expression in testis seems to be restricted to pachytene spermatocytes and post-meiotic spermatids. There are two classes of these proteins, carnitine palmitoyl transferase I and II. They are found on the external

and internal surfaces of the inner mitochondrial membrane, with type I on the cytosol surface and II on the matrix surface. They facilitate transport of carnitine into and out of the mitochondrial matrix. Carnitine palmitoyl transferases transfer the long chain fatty acid of acetylCoA to carnitine in the cytosol. The carnitine then enters the mitochondria and the fatty acid chain is removed from the carnitine and transferred to a CoA in the mitochondrial pool. The mitochondrial pool then functions in the oxidative degradation of pyruvate and certain amino acids including isoleucine, methionine, and valine. It was a surprise to identify the transcript for this protein in this screen, since we expect it would be playing a "house-keeping" role, with no specific function in spermatogenesis (Finocchiaro et al., 1990, Lesniak et al., 1995). However, it could be related to dramatic changes in mitochondrial structure and function that occur in spermatogenesis.

Alanyl tRNA synthetase is another sequence identified by the screen and database search. Aminoacyl tRNA synthetases allow coupling of the adenylated, activated amino acid to its appropriate tRNA. Thus this is a house-keeping protein which we presume is needed for the active protein-synthesis during spermatogenesis (Buechter and Schimmel, 1993). This gene appears to be expressed during all stages of spermatogenesis.

Many growth factors are found in testes. They act as autocrine and paracrine modulators of male gonadal function, but their precise role in spermatogenesis has not been defined. Insulin-like growth factor or ILGF is

believed to stimulate spermatogonial DNA synthesis and may have a maintaining effect on premeiotic DNA synthesis (Soder et al., 1992). Consistent with this view, we find it expressed in premeiotic, meiotic, and post-meiotic germ cells.

Novel Sequences

We also detected new, or unknown sequences in our BLAST analysis, and two blots which are representative of the different expression patterns were shown in Fig. 2.11. There are some interesting patterns of expression seen here. Four of the novel clones (1, 8, 13, and 17) are apparently testis-specific. These sequences are interesting clones to follow up by isolating full-length cDNAs and doing more sequence analysis. Some of the clones (7 and 16) are not testis specific.

Sex-LInked Genes

Two of the most interesting banding patterns discovered by Northern analysis are those of *Ott* and *Rbm*. Both of these genes reside on the sex chromosomes. *Ott* has been shown to be on the X chromosome and *Rbm* has been mapped to the Y chromosome. On cell-specific blots, both of these genes showed increased expression in the pachytene substage. This finding is unexpected because during the P substage the sex chromosomes are thought to be transcriptionally inactive, and therefore should not show increased expression of transcription at this time. Because of this unanticipated pattern of expression,

these sequences were investigated more thoroughly (Ch. 3).

Ott or the "ovary-testes transcribed" transcript is a mouse multigene family. This gene family maps to a small region on the X chromosome. Oddly enough, the *Ott* transcript was found in high levels in pachytene cells, where the X chromosome is transcriptionally inactive. However this may be due to stabilization of transcript synthesized before the chromosome becomes inactivated (Kerr et al., 1996).

Rbm, encoding an RNA-binding motif protein, is a gene located on the Y chromosome. It has been found in several copies in the mouse. It is in or near the AZF; or azoospermia factor region of the Y, and is thought to be important in the control of spermatogenesis. Men lacking this gene have low sperm count or no sperm at all. If this gene does control post-meiotic differentiation of sperm, this might account for its expression (Chandley and Cooke, 1994, Eberhart et al., 1996, Elliot et al., 1996, Ma et al., 1993, Maiwald et al., 1996, Reijo et al., 1995, Vogt et al., 1995).

Conclusions

It is exciting that our screening process identified so many clones. The 18 clones we have analyzed here are only the beginning of what we can learn using the results of library screening. However it was disappointing that our homology search did not give us any transcripts with homology to proteins known to be required for meiosis in other species, like recombination or synaptonemal

complex proteins. It is likely that such sequences would be expressed primarily early in meiotic prophase, perhaps even in the T38 mutant used in the first screening step, and thus not detected by this screen. We did identify some putative house-keeping genes that may also play a role in spermatogenesis, but most of these appear to be ubiquitously expressed. It is not yet known if the novel proteins we have detected are meiosis or spermatogenic specific. Thus the novel sequences revealed will be of considerable interest. Finding full length sequence, more detailed expression pattern, genetic mapping, and function during meiosis for these cDNAs, especially the four testes-specific ones, may identify some interesting proteins. The four clones that show expression restricted to meiotic or post meiotic germ cells may represent proteins whose function is specific to and/or essential for meiosis. Clearly however, from the results of this screen meiotic gene expression encompasses not only genes required for meiosis but also genes whose products are required for the ensuing spermiogenic differentiation.

The identification of *Ott* and *Rbm* gene transcripts through this screen was interesting also. Their gene expression will be considered in more detail in the following chapter. If these genes are expressed, either specifically (as with *Ott*) or increased at this substage (*Rbm*), it will be interesting to discover if they are being transcribed during the P substage, what role in meiosis they actually play, and why they are transcribed when other genes on the sex chromosomes are silenced.

CHAPTER III: Analysis of Sex Chromosome Expression in Meiosis

BACKGROUND AND SIGNIFICANCE

As described in chapter 2, two sex-linked genes were identified by the screening process of Caldwell *et al.* (1996). This was unexpected because the sex chromosomes are thought to be inactive during meiotic prophase. The two clones identified are *Ott* and *Rbm*. The purpose of this study is to examine the expression and nuclease sensitivity of these two genes.

One important issue is the relationship of gene recognition and expression to chromatin configuration. There are many examples of chromatin conformation playing a role in recombination events. It is known to be involved in initiation of yeast mating-type switching (Nasmyth, 1982) and in initiation of recombination at the mouse *Eb* meiotic recombination hotspot (Shenkar et al., 1991). In both yeast (Stapelton and Petes, 1991) and mice (Shenkar et al., 1991) hotspots for meiotic recombination have been mapped to sites accompanying gene promoters or enhancers. In yeast all double strand break point regions inspected so far have been found to lie in regions that are DNase sensitive. This has not been studied in mammals, although a few recombination hotspots have been found in mammalian systems. The state of chromatin is viewed as being a likely factor in controlling the interaction of recombination machinery with its initiation sites. It is thought that the more condensed the

chromatin, the less likely recombination factors will be able to interact with the chromatin and cause recombination. In mammalian spermatocytes the condensation states of the sex chromosomes versus the autosomes is very different. The autosomes are less condensed and more DNase sensitive than the sex chromosomes (Wiltshire *et al,* in press). The state of condensation could be indicative of either the transcriptive or recombination activity of the different types of chromosomes. Wiltshire *et al.* (in press) found that, in spermatocytes, DNase sensitivity was more closely correlated with potential for recombination than it was with transcription. Perhaps sex chromosome condensation may not occur to prevent transcription of X-linked genes inhibitory to spermatogenesis, but may serve instead to prevent recombination, with transcriptional inactivation as a secondary effect.

Condensation of the sex chromosomes in spermatocytes occurs during the pachytene substage of prophase I, when autosomes are undergoing recombination. Sex chromosomes condense into a structure called the sex body, sex vesicle, or X-Y body (Solari, 1989), and the recombination between these chromosomes is limited to a small terminal region of each of the sex chromosomes. Because the Y chromosome contains genes encoding proteins needed for determination of the male sex, if recombination were to occur between these chromosomes, the results could be detrimental to normal sex determination. Aside from the differences between autosomes and sex chromosomes during the pachytene stage of spermatogenesis, there are also differences in the behavior of the sex

chromosomes during male meiosis versus female meiosis. In oogenesis, the inactive X is reactivated, so both X chromosomes are transcriptionally active and recombination may occur along the entire length of the chromosomes. In spermatogenesis, the sex chromosomes are transcriptionally inactive, and recombination occurs only in the small, terminal region at which they are paired. McKee and Handel (1993) suggested that reactivation of the X in female meiosis permits recombination between the two X chromosomes during oogenesis and that the X-Y body forms in male meiosis so that recombination cannot occur between the heteromorphic sex chromosomes.

In view of these data, it was surprising to detect two sex-linked genes in a screen for pachytene expressed sequences (Caldwell *et al.*, 1996). *Ott*, or "Ovary-testes transcribed" transcript, is expressed specifically in these two tissues, and is an X linked multigene family. Kerr *et al.* (1996) reported two different *Ott* transcripts in the mouse testis. One transcript is expressed throughout meiosis and is believed to be a polyadenylated transcript of approximately 2.3 kb. The other transcript is expressed in the testis after four weeks of age and is approximately 1.0 kb. *Ott* is proposed to be transcribed from at least 7 different genes located on a small region of the X chromosome.

Rbm ("RNA binding motif") is a Y linked gene which is thought to play an important role in spermatogenesis. It is located in the AZF, or azoospermia, region of the Y chromosome, and has been found to be present in several copies in the mouse. Men lacking the human homolog of this gene have low sperm count or

no sperm at all. (Chandley and Cooke, 1994, Eberhart et al., 1996, Elliot et al., 1996, Ma et al., 1993, Maiwald et al., 1996, Reijo et al., 1995, Vogt et al., 1995).

Since finding *Ott* and *Rbm* clones among sequences identified by our screen (Caldwell *et al*, 1996) was unexpected, it seemed important to learn more about their expression during prophase I. Determining exact expression patterns on cell specific blots (CSBs) and expression in our induced metaphase system using okadaic acid were the first steps in ascertaining what these genes may be doing during this phase of meiosis.

MATERIALS AND METHODS

Isolation of Spermatogenic Cells

Germ cells were prepared from 8-day- old mouse testes (for type A and type B spermatogonia), from 17-day-old mouse testes (for leptotene/zygotene cells), and from adult mouse testes (to yield adult pachytene spermatocytes, round spermatids and residual bodies). Testis tubules were suspended in KRB [120.1 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 1.3 mM $CaCl_2$, 11 mM glucose, 1X essential amino acids (Sigma, St. Louis, MO), 1X non-essential amino acids (Sigma, St. Louis, MO)] and were digested with collagenase, trypsin and DNase as previously described (Romrell *et*

al., 1976; Bellvé *et al.*, 1977). After tubule digestion, cells were washed with 0.5% bovine serum albumin to remove trypsin and collagenase. Cells were then separated by sedimentation on a bovine serum albumin gradient at unit density using the STA-PUT chamber (Johns Scientific, Ontario). After 2.5 hours, cells were collected in 10 ml fractions at a rate of 10 ml/43 seconds. Cell fractions were assessed for morphology and purity by differential interference contrast (DIC) optics and enriched fractions pooled. Each cell type was enriched to a purity of >80%. Type A and type B spermatogonia, as well as leptotene/zygotene spermatocytes were obtained using 65 mice of the appropriate age. Pachytene spermatocytes, round spermatids, and residual bodies were isolated using 10 adult mice.

Isolation of Total RNA from Germ Cells

Total RNA was isolated from germ cells by the method of Cathala *et al.* (1983). Diethylpyrocarbonate-treated glassware and solutions were used in all steps. Purified germ cells were frozen at -80° overnight, and were then thawed in guanidinium isothiocyanate (5 M guanidinium isothiocyanate, 10 M EDTA, 50 mM Tris-HCl pH 7.5) and sheared with a 20 1/2 gauge syringe 20 times. The RNA was precipitated overnight with 7 volumes 4 M lithium chloride at 4°. Samples were then centrifuged at 4° for 1.5 hours at 10,000 x g and the pellets were resuspended in RNA solubilization buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% SDS). RNA was extracted with phenol followed by chloroform and

then precipitated with sodium acetate (0.2 M NaAc) and 100% ethanol overnight. Samples were then centrifuged for 10 minutes at high speed in the microcentrifuge and the pellets were resuspended in 200 µl depc-water and were quantitated by spectrophotometry. In addition to preparing RNA from specific germ cell types, RNA was isolated by the same methods from whole testes of adult mice (WT) and from testes of genetically germ-cell-deficient mice (XX, *Sxr*). Mouse somatic tissue RNA was obtained from Ambion (Austin, TX).

Northern Analysis

15 µg of total RNA from each sample (XX*Sxr,* WT, A, B, LZ, P, RS, and RB) was denatured in 50% formamide (Ambion, Austin, TX), 1X MOPS buffer, 6.5% formaldehyde pH 6.0, and Type III loading buffer [0.25% bromophenol blue, 0.25% xylene cyanol, and 30 % glycerol (Sambrook *et al.*, 1989)] for 15 minutes at 65°. The samples were then loaded onto a denaturing 1% agarose gel (1X MOPS buffer, 2.2 M formaldehyde). Samples were electrophoreses at 50V for 4.5 hours or until the dye had moved about 3/4 of the gel length. The gel was then washed twice in deionized water for 30 minutes, stained with ethidium bromide at 5µg/ml for 20 minutes, and destained for 20 minutes. The gel was photographed to visualize the ribosomal 28S and 18S bands. It was then soaked in 20X SSC (3M NaCl, 0.3M NaCitrate) and transferred overnight to nylon membrane (Sratagene, LaJolla, CA). RNA was crosslinked to the nylon blot at 1280 µjoules/cm^2 in a Stratalinker (Stratagene, LaJolla, CA).

Hybridization of Northern Blots

Prehybridization was in 5X SSPE, (3M NaCl, 0.02M EDTA, 0.1M NaH_2PO_4), 5X Denhardt's Solution (6.25nM Ficoll, 62.5nM polyvinylpyrrolidone, 1% BSA), 0.5% SDS, 0.25 mg/ml fish sperm (Boehringer Mannheim, Indianapolis, IN) at 65°C for 3 hours in a hybridization incubator (Lab-Line Industries). After removal of the prehybridization solution, fresh prehybridization solution was added with denatured probe at a concentration of 1 million cpm/ml. Probes were made from clones in Bluescript plasmid (Stratagene, LaJolla, CA); standard methods were used for isolating the inserts and radiolabelling by the random-priming method with α-32PdCTP (ICN, Costa Mesa, CA)]. Hybridization was at 65°C overnight in the incubator. Following hybridization the blots were washed twice with 1X SSC and 0.1% SDS at 65°C and the twice with 0.1X SSC at 65°C. Blots were wrapped in plastic and exposed to film (Reflections, ICN, Costa Mesa, CA) at -80°C for appropriate period of time. Stripping of blots was done using boiling SDS (0.25%) as recommended by the manufacturer (Stratagene).

Isolation of Plasmid Clones and Sequence Analysis

Clones were purified using Nucleobond AX-100 purification columns (The Nest Group, Macherey-Nagel). Aliquots of each clone were subjected to single-pass automated sequencing, conducted under the direction of Dr. J. Schimenti, The Jackson Laboratory. This yielded at least partial sequence for

each clone. Comparison to sequence data bases was performed using the BLAST algorithm (National Center for Biotechnology Information). BLAST analysis was performed on a regular basis throughout the study.

RESULTS

Cell Specific Northern Blots

Northern blots with various tissue and cell samples were probed for both *Ott* and *Rbm* and results are shown in figures 3.1 and 3.2.

As seen in Fig. 3.1 A, an *Rbm* probe detects a single band, found in all lanes except for that with RNA from XX,*Sxr* testes. Since the *Rbm* gene is located on the Y chromosome, it would not be expected to be in XX,*Sxr* mice which lack all but a small part of the Y chromosome. The single band is approximately 1.5 kb,which was expected from previously published results. The *Rbm* transcript is expressed highly in A spermatogonia and LZ spermatocytes and, as seen in the figure, is less highly expressed in the pachytene substage of meiosis.

The *Ott* transcript is detected in all lanes of these Northern blots. There appear to be five different isoforms of the transcript, with differential expression throughout prophase of meiosis. Formerly, only three isoforms had been reported in the mouse testis (Kerr *et al.* 1996). *Ott* transcript is expressed in all substages. Furthermore, it is detected in RNA from XX,*Sxr* testes, which tells us that this transcript is not germ-cell specific since these

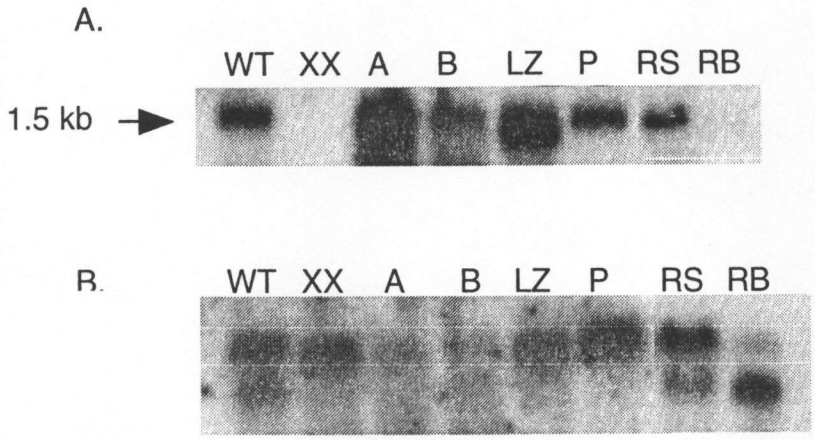


Figure 3.1 - Gene expression of *Rbm* on cell specific Northern blots. A. Autoradiograph of *Rbm*. B. Same cell-specific blot probed with actin

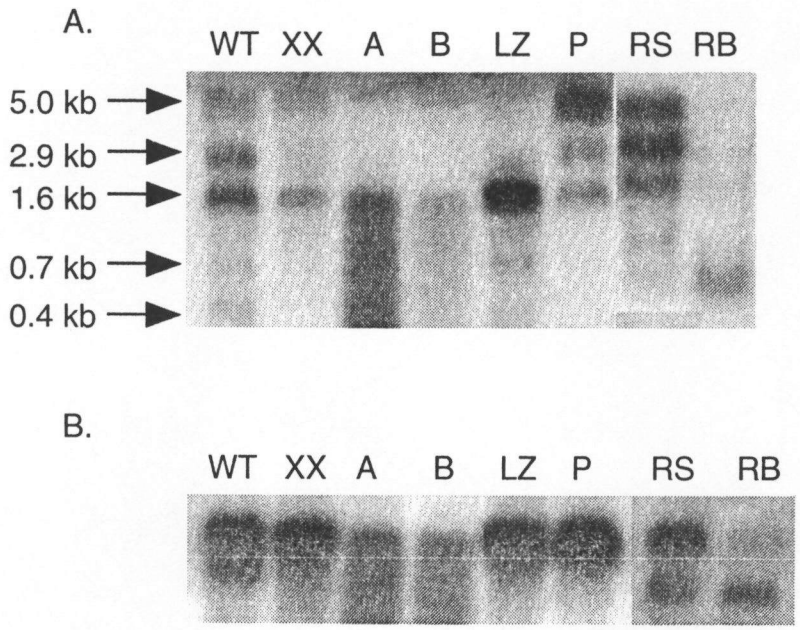


Figure 3.2 - Gene expression of *Ott* on cell specific Northern blots. A. Autoradiogram of *Ott* expression. B. Same cell-specific blot probed with actin.

mice are germ-cell deficient. Interestingly, one of the isoforms, 5.0 kb, shows increased expression in the pachytene substage, as opposed to LZ, when the sex chromosomes are thought to be inactivated.

Okadiac-Acid Treated Pachytene Spermatocyte Northern Blots

RNA isolated from cells treated with OA for zero and six hours was Northern blotted to membrane. These membranes also contained RNA from cultured cells not treated with OA for six hours. Labeled probes of *Ott* and *Rbm* were used to probe these blots. In each case it was determined that inducing the cells to prematurely enter metaphase has no effect on expression of these transcripts.

Rbm and *Ott* both show the same banding patterns on these blots (figure 3.3 A and B) as they do in pachytene cells (Fig. 3.1 and 3.2). There is no increase or decrease of expression in any of the lanes.

DISCUSSION

Cell-Specific Northern Analysis

The expression pattern of *Rbm* was what we expected a sex-linked gene to yield. It is expressed highly in leptotene/zygotene spermatocytes but its expression decreases in pachytene spermatocytes. It therefore, may not be being actively transcribed in the pachytene substage, when the X-Y body is present,

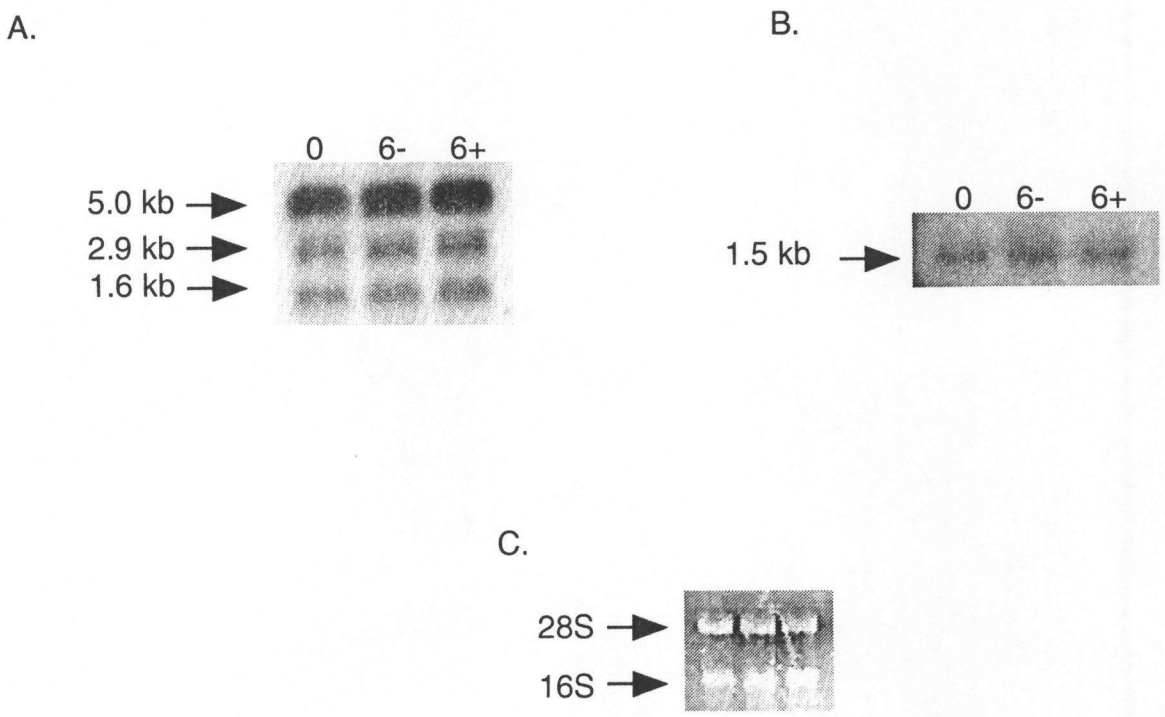


Fig. 3.3 - Analysis of gene expression of Ott and Rbm during induced prophase of meiosis by OA. Lane 1- pachytene spermatocytes treated with OA for 0 hours, lane2- pachytene spermatocytes cultured for 6 hours without OA, and lane 3- pachytene spermatocytes treated with OA for 6 hours. A. Autoradiograph ot the blot probed with Ott and B. probed with Rbm. C. Ethidium bromide stained agarose gel.

but is most likely being transcribed earlier in meiosis.

The expression pattern of *Ott* is a little more puzzling. It is interesting that there appears to be a different isoform being expressed highly in pachytene spermatocytes, but at low levels in leptotene/zygotene spermatocytes. It also appears that this isoform may be germ-cell specific as it is not seen in the XX,*Sxr* lane. Because the sex chromosomes are thought to be transcriptionally inactive during the pachytene substage of meiosis, this expression is interesting. It is possible that the transcription is occurring late in zygotene, and is being stabilized in pachytene but we would have expected to see greater intensity in the leptotene/zygotene spermatocytes if this were true. Because this transcript has a higher molecular weight than those seen in earlier substages, it does not appear to be a degradation product of the gene transcribed earlier. It is possible that this isoform could be an alternative processing product of a common precursor transcribed earlier, possibly an autosomal member of the gene family, or that the transcript has been stabilized during the transition from leptotene/zygotene to pachytene.

Although this pattern of *Ott* transcript differs from the previous one we determined and showed in chapter two, we believe it to be the accurate pattern. The difference between the two is probably due to errors during washing of the blots. When doing the work for the previous chapter, our Geiger counter was not working and would read that everything was radioactive. We were misled and the blots were washed under overly stringent conditions, no doubt leading to loss of

bands. We believe that the band seen in pachytene spermatocytes on previous blots is the higher molecular weight band seen on this blot.

Northern Blots of Okadaic Acid Treated Spermatocytes

We have used okadaic acid to prematurely induce pachytene spermatocytes to enter the metaphase I stage of meiosis (Wiltshire *et al.*, 1995) and show that pachytene spermatocytes are capable of entering the next stage of meiosis in *vitro* earlier than they actually do *in vivo*. The expression patterns revealed by these blots appear to be exactly the same as those seen in pachytene spermatocytes from the cell-specific Northern blots, therefore these two genes do not appear to vary in expression during the prophase I-metaphase I transition in this inducible model. (It is possible that the isoform of *Ott* that appears in pachytene spermatocytes is needed later during spermiogenesis rather than in meiosis.)

CHAPTER IV: Conclusions and Future Directions

Although meiosis is an important process, little is known about gene expression during this time. The main goal of both of the chapters presented here was to identify and analyze genes expressed during spermatogenesis. From a prior screening (Caldwell et al., 1996) 18 clones were isolated; they are assumed to be expressed in the window of time between late leptotene/zygotene and pachytene. Of those 18 sequences, we here identified six as novel sequences, showing little homology to known sequences in informatics databases. Six of the other sequences were found to be homologous to house-keeping genes (carnitine palmitoyl transferase, alanyl tRNA synthetase, calmodulin, ILGF binding protein, thymosine ß, and alpha tubulin) and two were identified as sex-linked genes (*Ott* and *Rbm*).

Of the novel sequences, four clones (1, 8, 13, and 17) showed expression only in the testis and one clone showed expression restricted to the testes and the brain (6), on tissue-specific Northern blots. We believe these clones would be interesting targets for further analysis. To re-evaluate their cell-specific expression patterns with Northern analysis and possibly determine when during prophase they are being transcribed with nuclear run-off assays may be the first step in identifying the role of these gene during this important process.

Because these analyses simply tell when the genes are transcribed, and

tell nothing about the role these genes play in meiosis, experiments need to be done to determine this information. Developing knockout mouse strains, each lacking one of these genes, is one way to identify the gene's specific role in this process. However, if a phenotype is not observed (ie. no halt in spermatogenesis), sometimes identifying the role of the gene becomes laborious using this technique. Yet even if a phenotype is identified, this is only the first step in finding the gene's function. Discovering if the phenotype is a primary or secondary effect of the absence of the protein can be a painstaking process.

Other ways to discover a gene's role may be by identifying proteins with which the gene interacts. Protein immunoprecipitation assays and yeast two hybrid assays may be utilized for this purpose. If the roles of interacting proteins are known, this may help in detecting what is the function of our protein. Fluorescent antibodies can be used to determine where a protein may be localized to during the specific substages of meiosis. If fluorescent antibodies are used to determine cell localization within a substage, this may give information as to what the protein of interest may be doing.

We were surprised to identify the two sex-linked genes through this screen. Because the X and Y chromosomes are thought to be transcriptionally inactive during the pachytene substage of meiosis in males, we did not expect genes located on either of these chromosomes to appear. Therefore, they became interesting for further study. We then did another set of cell-specific Northern blots to ascertain that we had the correct expression patterns for each. Although

the expression pattern for *Rbm* remained the same, it was surprising to learn that the *Ott* banding pattern previously determined was incorrect. We believe this is due to stringent washings, and the fact that our Geiger counter was not working properly. On these blots, it was discovered that *Ott* has at least three isoforms expressed during spermatogenesis. The most interesting discovery was that one isoform is expressed most highly during the pachytene substage. This was surprising because during this time the X and Y chromosome are thought to be transcriptionally inactive.

Next we wanted to determine if the gene expression of either *Ott* or *Rbm* changed in our metaphase I-induced cell culture system. When okadaic acid is added to cultured pachytene cells *in vitro*, these cells enter metaphase I earlier than *in vivo*. Therefore, Northern blots were made with pachytene cells treated with okadaic acid for various time points, and these blots were hybridized with *Ott* and *Rbm*. There was no change in gene expression patterns seen on blots of okadaic acid treated cells. Thus, there appears to be no change in gene expression during the prophase I-metaphase I induced transition.

DNase sensitivity assays can be used to analyze chromatin conformation. Previous studies (Wiltshire *et al.*, in press) have shown that in general, gene sequences on autosomal chromosomes are more DNase sensitive than are gene sequences on sex chromosomes. Experiments were begun to determine if the *Ott* gene was as sensitive to nuclease digestion as another sex-linked gene, *Hprt*. We were unable to determine this due to technical problems with Southern blots. If

it is discovered that *Ott* is more DNase sensitive than *Hprt* , this could be the determining factor in whether or not to continue with experiments to find out when during meiotic prophase *Ott* is being transcribed. Although chromatin conformation has been correlated to recombination in spermatogenesis (Wiltshire *et al.*, in press), these assays could aid in determining if nuclear run-off assays or experiments determining stabilization states of genes should be done to learn the substage of meiosis the time of transcription.

During the screening process done by Caldwell *et al.* (1996), groups of clones were identified other than the ones we have worked with here. One of these groups consisted of genes expressed in cDNA isolated from T38 testes which halt during late leptotene/zygotene (referred to as T38 clones). Therefore, these genes are expressed during L/Z and earlier. Because we did not identify any genes homologous to known meiosis genes (such as synaptonemal complex or recombination proteins) in the group we worked with here, we believe these genes may be being transcribed earlier than this window of time between L/Z and P, possibly in late spermatogonia or early leptotene/zygotene. It has been found that genes have been transcribed in earlier substages of meiosis but not translated until postmeiotic substages, as with thymosine ß (Lin and Morrison-Bogorad, 1991). If similar analyses were done with the T38 clones as were done with the clones presented earlier in this paper, we may identify genes necessary for recombination or synaptonemal complex formation. Another way to go about identifying genes important for meiosis may be to start with known homologs in

other species and probe our cDNA libraries with these or to use antibodies to known meiosis genes and first determine in which substage the protein is present and then determine when transcription is occurring.

Identifying genes known to be involved in meiosis either by studying T38 clones or continued study of the novel gene sequences identified as testes-specific, as well as learning about the roles of *Ott* and *Rbm* during spermatogenesis is important to understanding more about gene expression during this vital process.

We believe the information we have gained from the work presented here is important although not what we intended to determine. We have discovered that gene expression patterns during meiosis are complex and diverse, and that there are many types of genes being expressed during this time, many of which probably play little or no role in meiosis at all. We do feel that if we were to continue this type of work we would not continue working with all of these 18 clones. We would continue to work with the four novel testes-specific clones as well as to similarly analyze the T38 group of clones in an attempt to identify homologs of known meiosis "necessary" genes in other species, and if this did not work would then use the known genes as probes in our libraries.

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